

NASA-CR-204714

## Brief Report

# Tissue-Engineered Skeletal Muscle Organoids for Reversible Gene Therapy

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### ABSTRACT

Genetically modified murine skeletal myoblasts were tissue engineered *in vitro* into organ-like structures (organoids) containing only postmitotic myofibers secreting pharmacological levels of recombinant human growth hormone (rhGH). Subcutaneous organoid implantation under tension led to the rapid and stable appearance of physiological sera levels of rhGH for up to 12 weeks, whereas surgical removal led to its rapid disappearance. Reversible delivery of bioactive compounds from postmitotic cells in tissue engineered organs has several advantages over other forms of muscle gene therapy.

### INTRODUCTION

**S**KELETAL MUSCLE IS A PRIMARY TARGET for the delivery of systemic therapeutic agents through gene therapy (Blau and Springer, 1995b) because of its capacity to express foreign DNA either injected directly as plasmid DNA (Wolff *et al.*, 1990), or injected as *ex vivo*-modified myoblasts (Barr and Leiden, 1991; Dhawan *et al.*, 1991). In both instances, the DNA is incorporated into the host's postmitotic myofibers and results in the long-term expression and delivery of soluble, bioactive compounds to the circulation. Myoblast gene therapy has successfully delivered factors such as growth hormone (Barr and Leiden, 1991; Dhawan *et al.*, 1991), factor IX (Yao and Kurachi, 1992), and erythropoietin (Hamamori *et al.*, 1994, 1995), whereas direct intramuscular injection of plasmid DNA containing the sequence for kallikrein (Xiong *et al.*, 1995) or kallikrein-binding protein (Ma *et al.*, 1995) leads to the systemic delivery of these blood pressure-regulating molecules. Several problems currently exist with these gene therapy protocols. First, the *in vivo* incorporation of injected plasmid DNA and myoblasts into host myofibers is variable and inefficient (Acsadi *et al.*, 1991; Huard *et al.*, 1995), making delivery of a predetermined level of a bioactive compound difficult. Second, the foreign DNA can remain dormant for extended periods *in vivo* (Yao *et al.*, 1994), or can migrate through the vascular sys-

tem to other sites (Neumeyer *et al.*, 1992). Subsequent surgical removal of the foreign DNA-containing tissue, if necessary, would thus be difficult.

Tissue engineering is a new discipline for the *in vitro* construction of varied tissues such as pancreatic islets, liver, skin, cartilage, bone, muscle, and blood vessels (Langer and Vacanti, 1993). Although the primary goal of tissue engineering is to replace the function of a deficient organ, engineered tissues may also serve as a vehicle for gene therapy. Skeletal muscle myoblasts can be tissue engineered *in vitro* into organ-like structures (organoids) containing parallel, postmitotic myofibers connected to tendon-like structures and organized into fascicles, the functional unit of muscle (Vandenburg *et al.*, 1991). Use of these tissue-engineered skeletal muscle organoids for the systemic delivery of bioactive compounds from foreign DNA would have advantages over other muscle delivery systems, including the highly efficient *in vitro* fusion of myoblasts into postmitotic myofibers, implantation of only postmitotic cells, preimplantation monitoring of organoid secretory levels of the bioactive compound, and reversibility by surgical intervention.

In this report, we describe the use of tissue-engineered skeletal muscle organoids for systemic delivery of a bioactive compound. Skeletal muscle organoids containing only postmitotic myofibers were formed *in vitro* from genetically modified

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murine myoblasts secreting recombinant human growth hormone (rhGH; Dhawan *et al.*, 1991). When implanted subcutaneously into syngeneic mice, the organoids delivered constant physiological levels of rhGH from the day of implantation out to 12 weeks. Surgical removal of the implant was easily accomplished, and rhGH levels were rapidly decreased. Similar combined tissue engineering-gene therapy techniques may find applications in other organs, including bone, lung, blood vessels, skin, and the heart. Part of this work has appeared in abstract form (Vandenburg *et al.*, 1996).

## MATERIALS AND METHODS

### *Tissue engineering murine C2C12 rhGH skeletal muscle organoids*

Murine C2C12 skeletal myoblasts (C2MIXAB) stably transduced with the rhGH gene (Dhawan *et al.*, 1991) were a gift from Dr. Helen Blau (Stanford University, CA). The rhGH gene is under control of the retroviral long terminal repeat (LTR) promoter. The cells also contain the *lacZ* gene for bacterial  $\beta$ -galactosidase ( $\beta$ -gal) under control of the cytomegalovirus (CMV) enhancer/ $\alpha$ -globin promoter, allowing histological localization of the transduced cells. Nontransduced control C2C12 myoblasts were obtained from the ATCC (Rockville, MD, cat no. CRL1772). The cells were grown in tissue culture dishes in growth medium C2GM: Dulbecco's modified Eagles' medium (DMEM) with high glucose (Life Technologies, Inc., Grand Island, NY), 5% fetal calf serum (FCS; Sigma Chem. Co., St. Louis, MO), 15% defined supplemented calf serum (Hyclone Laboratories, Inc., Logan, UT), penicillin (100 units/ml, Sigma Chem. Co.), and streptomycin (0.1 mg/ml, Sigma Chem. Co). Greater than 99% of the C2MIXAB myoblasts were positive for  $\beta$ -Gal, determined by  $\beta$ -Gal histological staining. For organoid formation, myoblasts were harvested with 0.05% (wt/vol) trypsin, 0.02% (wt/vol) EDTA in  $\text{Ca}^{2+}$   $\text{Mg}^{2+}$ -free Earle's balanced salt solution, and pelleted by centrifugation at 1,200 rpm for 4 min. The cells were resuspended in an ice-cold solution of C2GM containing 1.6 mg/ml collagen (Type I rat tail, Collaborative Biomedical Products, Bedford, MA) neutralized to pH 7.1 with sterile 0.1 N NaOH as previously described (Vandenburg *et al.*, 1988). An ice-cold solution of MATRIGEL (Collaborative Biomedical Products) was added to the cell suspension (1:6, vol/vol), and the mixture was immediately cast in molds (0.4 ml/mold containing  $1-4 \times 10^6$  cells) made from silicone rubber tubing (4.8 mm i.d.) cut lengthwise into 30-mm lengths. Each end of the mold contained a 3-mm  $\times$  4-mm piece of either Velcro or stainless steel screening for organoid attachment (Vandenburg *et al.*, 1991). The molds were glued to the bottom of 35-mm tissue culture dishes. Following casting, the molds were incubated at 37°C for 2-4 hr to solidify the gel. The gels were then covered with 4 ml of C2GM, incubated for 3-5 days, and the medium was changed to a myoblast fusion medium for 4 days consisting of Dulbecco's modified Eagle's medium (DMEM) with high glucose, 2% horse serum (Sigma Chem. Co.), 100 units/ml penicillin. Fusion efficiency in this medium is 75-80%, as determined by nuclear counting of hematoxylin-eosin stained cultures (data not shown). After 1 to 2 days *in vitro*, the gel de-

tached from the silicone rubber mold, and was held at its ends by the Velcro or stainless steel screening. The gel contracted to form a cylindrical structure approximately 2.5 mm in diameter by 18 mm in length. After 4-5 days in fusion medium, the organoids were switched to a maintenance medium (DMEM with high glucose, 10% horse serum, 5% FCS, and 100 units/ml penicillin), and treated with 1  $\mu\text{g/ml}$  cytosine arabinoside (araC) for 4-6 days to remove nonfused proliferating cells. The organoids were maintained in this maintenance medium for 5-12 days before implantation.

### *Surgical implantation of muscle organoids*

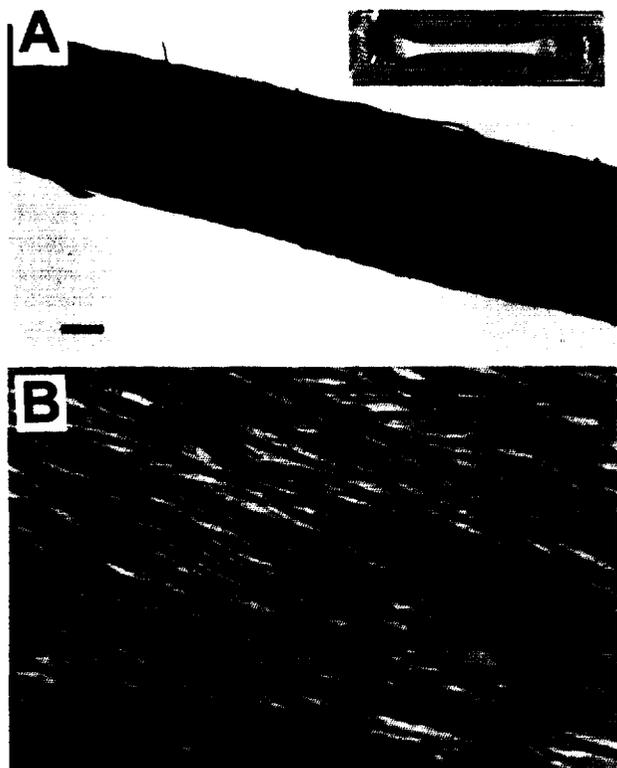
Before surgical implantation, the organoids were preincubated at 37°C in DMEM containing 0.125% (wt/vol) bovine serum albumin for 1 hr. The sides of the rubber mold containing the organoid were removed with a scalpel so that the organoid remained attached at its ends in the bottom of the mold and under tension when implanted. All experimental animals procedures were approved by the Institutional Animal Care and Utilization Committee and conformed to the guiding principles of the American Physiological Society. The breeder stock of C3HeB/FeJ mice was obtained from Jackson Laboratory (Bar Harbor, ME). The animals were anesthetized with a mixture of ketamine (55 mg/kg), promazine (1 mg/kg), and xylazine (5 mg/kg), their backs were shaved and sterilized. A 20- to 30-mm incision was made along the mid-line, the skin was reflected, organoids were inserted under the skin flap, and the wound sutured closed. Implantation of the organoids is a rapid procedure (<10 min) and up to four implants can be inserted into each animal. The mice were injected with 60 mg/kg cyclosporine A at least 1 hr before surgery and daily thereafter to suppress antibody production to rhGH (Behara *et al.*, 1992). Levels of rhGH were greatest in animals receiving multiple implants, but animal survival was decreased. For the single organoid implants used in the present study, animal death over 30 days averages 50%. This results from cyclosporine A toxicity, because mortality is the same in no-implant cyclosporine A-treated controls (data not shown).

### *rhGH radioimmunoassay*

A sensitive radioimmunoassay exists for rhGH, which does not cross-react with murine growth hormone (GH) (Barr and Leiden, 1991; Dhawan *et al.*, 1991) (Nichols Diagnostic Lab, San Juan Capistrano, CA). The assay is accurate and linear from 0.25 ng/ml to 50 ng/ml. Tissue culture medium aliquots were assayed directly after a 500-fold dilution. Because C2C12 cells do not metabolize rhGH in tissue culture (Barr and Leiden, 1991), medium levels represent accumulated rhGH secretion. In contrast, rhGH turns over rapidly *in vivo* (half life <10 min; Strobl and Thomas, 1994), and undiluted serum rhGH measurements from animal tail bleeds serve as a real-time assay for rhGH secretion from the implants.

### *Histological staining techniques*

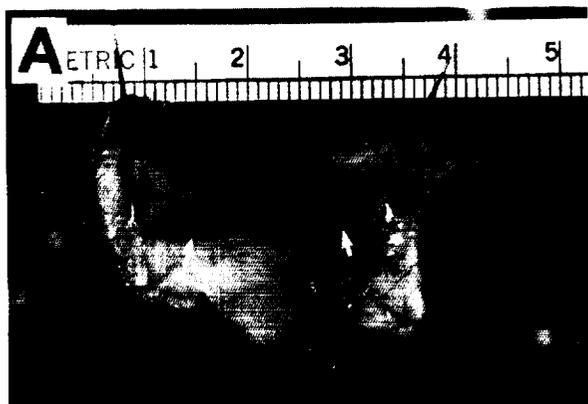
The differentiated organoids were fixed in 1 $\times$  HISTO-CHOICE (Amresco, Solon, OH) for 15-30 min, rinsed with phosphate-buffered saline (PBS), and immunologically stained (Vandenburg *et al.*, 1991) with an antibody against sarcom-



**PLATE 1.** Tissue-engineered skeletal muscle organoids with organized myofibers. C2MIXAB myoblasts suspended in an extracellular matrix gel are cast in silicone rubber molds and grown under tension for 2–3 weeks *in vitro*. Low-power (A) and high-power (B) whole-mount photographs show the longitudinal alignment of sarcomeric tropomyosin-stained myofibers. The inset in A is a 48-hr postplating unstained organoid in the casting mold and attached at its ends to stainless steel screening. Detachment of these organoids at one end causes them to contract to one-third their length within 12 hr, indicating the large internal tensions in the tissue. Bars represent 250  $\mu\text{m}$ , 6 mm, and 20  $\mu\text{m}$  in A, A inset, and B, respectively.



**PLATE 3.** Post-mitotic myofibers in subcutaneously implanted muscle organoids survive *in vivo*. Muscle organoids implanted for 2 weeks *in vivo* are surgically removed, fixed in 0.25% buffered glutaraldehyde, embedded, and frozen in liquid nitrogen for cryostat sectioning. A. Ten-micrometer cryostat cross section of the organoid with large-well differentiated and oriented myofibers under a host-formed epimysium (arrow). Hematoxylin and eosin stained. B. Ten-micrometer cryostat cross section stained for  $\beta\text{-Gal}$  shows heavily stained myofibers also containing the rhGH gene. Bars in A and B represent 8  $\mu\text{m}$ .



**PLATE 2.** Delivery of rhGH from tissue engineered muscle organoids is a rapidly reversible procedure. A. A 40-day subcutaneous implanted organoid retains its morphologically distinct structure. The silicone rubber mold that holds the organoid under tension *in vivo* is removed for the photograph. The black Velcro end pieces to which the organoid is attached are indicated by arrows. The reddish color of the implant indicates that it is well vascularized. B. Six animals are implanted with rhGH-secreting muscle organoids and on days 6–7 post-implant, implants are surgically removed (at R on graph) from 3 animals. C on the graph is an animal implanted with a control non-rhGH-secreting C2C12 organoid. Each line represents an individual animal. Similar results were obtained in two additional experiments.

eric tropomyosin (Sigma Chem. Co.). This was followed by an avidin-biotinylated secondary antibody coupled to horseradish peroxidase (Vectastain, Vector Laboratories, Burlingame, CA) and development with diaminobenzidine to form a brown precipitate. For  $\beta$ -Gal staining, organoids were fixed for 10–15 min in 0.25% glutaraldehyde, rinsed with PBS, and incubated overnight at 4°C in 30% (wt/vol) sucrose in PBS. The organoids were placed in TISSUE-TEK OTC Embedding Medium (Miles, Inc., Elkhart, IN) and frozen to -20°C; 10- $\mu$ m cryostat sections were cut. A HISTOMARK X-Gal Substrate Kit was used according to the manufacturer's recommendations (Kirkegaard and Perry Laboratories, Inc. Gaithersburg, MD).

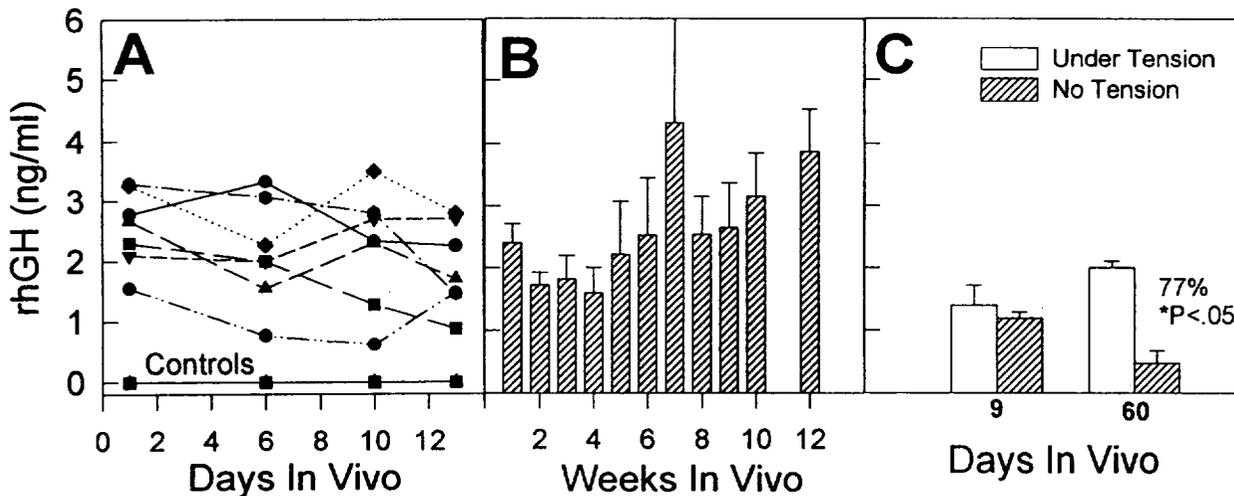
## RESULTS AND DISCUSSION

Skeletal muscle organoids are formed from C2MIXAB myoblasts after suspending in a solution of Type 1 collagen–MATRIGEL and casting the cell-gel mix in semicircular molds with the gross morphology of a skeletal muscle. High internal longitudinal tensions are generated in the gels as they dehydrate (Bell *et al.*, 1979), causing detachment from the mold surface and formation of a cylindrical structure attached only at its ends (Plate 1A, inset). These matrix tensions are adequate for aligning the fusing myoblasts into parallel arrays of myofibers expressing sarcomeric tropomyosin (Plate 1). Histological analysis of 2- to 3-week-old organoids shows a thick layer of well-differentiated, longitudinally oriented myofibers on the surface of the organoids, and a central core of extracellular matrix containing a low density of short two- to four-nuclei un-oriented myofibers (data not shown). The surface myofibers are

one to six cell layers deep ( $0.18 \pm 0.03$  mm thick), whereas the central connective tissue core is  $2.40 \pm 0.18$  mm in diameter (mean  $\pm$  SE,  $n = 4$ ). AraC ( $1 \mu\text{M}$  for 4–6 days) is effective in killing  $99.4 \pm 0.2\%$  (mean  $\pm$  SE,  $n = 4$ ) of the unfused C2MIXAB myoblasts, determined quantitatively on hematoxylin and tropomyosin-stained cultures (Vandenburg *et al.*, 1995).

The differentiated organoids secrete consistent levels of rhGH into their culture medium for several weeks, ranging from 1.0 to 2.9  $\mu\text{g}$  of rhGH/24 hr per organoid ( $n = 82$ ). This equals a production rate of 7–20 ng/10 min per organoid. Cytosine arabinoside treatment reduces rhGH secretion by 18% ( $p < 0.01$ ,  $n = 8$ ) as would be expected with a myoblast fusion efficiency of 80%, and equivalent levels of rhGH secretion per nucleus in unfused myoblasts and myofibers (Dhawan *et al.*, 1991). The rhGH secreted by the differentiated organoids comes from post-mitotic myofibers in which all of the myofiber nuclei contain the rhGH DNA, rather than a small subset of nuclei as occurs in the *in vivo* plasmid DNA and myoblast injection techniques.

Two- to three-week-old organoids secreting consistent amounts of rhGH are implanted (one/animal) under tension subcutaneously into the backs of 4- to 6-week-old C3HeB/FeJ mice syngeneic for the C2C12 cell line. In eight separate experiments, 32 of 45 animals survived the surgery for at least 72 hr. Of the 32 survivors, 27 produced constant physiological serum levels of rhGH (al-Hendy *et al.*, 1995), ranging from 0.5 to 3.5 ng/ml, from 24 hr to 10–14 days post-implantation (Fig. 1A). These rhGH levels are similar to those obtained with either direct intramuscular C2MIXAB myoblast injections (Dhawan *et al.*, 1991), or when rhGH-secreting primary fibroblasts are injected under the renal capsule (Heartlein *et al.*, 1994). Assuming



**FIG. 1.** Organoids containing post-mitotic C2MIXAB myofibers secrete physiological levels of rhGH when implanted under tension *in vivo*. A. Muscle organoids (one/animal) are implanted subcutaneously under tension into 4- to 6-week-old syngeneic HeB/FeJ mice at day 0 and sera levels of rhGH measured by radioimmunoassay (RIA). Each line represents an individual animal. Non-rhGH-secreting control organoids are formed from normal C2C12 myoblasts and implanted in an identical fashion. B. Weekly rhGH serum levels for animals implanted subcutaneously under tension with one organoid/animal. Values are means  $\pm$  SE for  $n = 25$  (week 1) to  $n = 5$  (week 12). There are no significant differences ( $p > 0.5$ ) between week 1 and week 12 values (paired *t*-test,  $n = 5$ ). C. Organoids implanted under no tension (day 0) produce similar levels of rhGH to organoids implanted under tension for 9 days *in vivo*, but by 60 days the secretion levels from no-tension implants are decreased compared to organoids implanted under tension. Bars are means  $\pm$  SE. A second experiment of this type gave similar results.

a mean half-life of 10 min for rhGH *in vivo* (Strobl and Thomas, 1994), it can be calculated that 28–30% of the rhGH produced by the organoids *in vitro* appears in the blood within 24 hr of implantation. These results imply that subcutaneous organoid implantation is an efficient site for rhGH delivery. Organoids continue to produce consistent levels of rhGH for 10–12 weeks *in vivo*, without any significant change from the secretion levels seen at 1 week (Fig. 1B). Week-to-week variations in rhGH levels were noted in the same animals (approximately two- to three-fold, *e.g.*, closed circle-solid line animal in Plate 2B) from 2 weeks to 12 weeks post implantation and may result from host regulation of rhGH transcription, translation, and/or secretion from the muscle fibers (Crystal, 1995). The consistent release of rhGH from the organoids is in marked contrast to rhGH secretion resulting from intramuscularly injected C2MIXAB myoblasts, where a 10- to 15-fold variation in serum rhGH levels occurs during the 12 weeks following injection (Dhawan *et al.*, 1991). The delivery of rhGH from organized postmitotic myofibers also appears to have advantages over its delivery from collagen-embedded proliferating, genetically modified fibroblasts, where reduced secretion with time results in a 95% loss in serum levels by 42 days *in vivo* (Chen *et al.*, 1995).

Maintaining the implanted organoids under tension is important for optimal long-term secretion of rhGH. Implantation of organoids under no tension results in a 77% reduction ( $p < 0.05$ ) in serum rhGH levels after 60 days *in vivo* compared to organoids implanted under tension (Fig. 1C). Loss of tension on organoid myofibers *in vitro* leads to significant myofiber atrophy, indicated by a 35% loss in organoid cellular proteins after 6 days of tension release ( $n = 4$ ,  $p < 0.01$ ). It has been reported by others that injection of myoblasts and disorganized myofibers provides a better bioactive compound delivery system than injection of myoblasts only (Jiao *et al.*, 1993), possibly because of the greater protein synthesis capacity of postmitotic muscle fibers compared to myoblasts. Prevention of myofiber atrophy in organoid structures by maintaining them under tension following implantation as done in the present study would further enhance the myofibers' large protein synthetic capability.

The systemic delivery of rhGH from the skeletal muscle organoids is a reversible procedure. For certain cell-based delivery techniques such as for bone repair (Fang *et al.*, 1996) or wound healing (Blau and Springer, 1995a), implant removal after healing may be desirable. The muscle organoids are rapidly encapsulated by host fibroblasts and vascularized when implanted subcutaneously, but they maintain their gross *in vitro* structure separate from the host (Plate 2A), and are easily removed surgically. Animals from which organoids are removed shows a rapid decrease in sera rhGH (Plate 2B). Hematoxylin and eosin staining of cryostat sections of the removed organoids shows a host-generated epimysium containing numerous fibroblast-like cells (Plate 3A). Capillaries are noted in the epimysium but not in the central connective tissue core of the organoid (data not shown). The myofibers underneath the epimysium are well-differentiated and maintain their parallel orientation. When cryostat sections of the removed organoids are histologically stained for  $\beta$ -Gal to identify the implanted myofibers, only well-aligned myofibers are positively stained (Plate 3B). No  $\beta$ -Gal-positive, proliferating cells or tumor-like structures are seen in

the mice, as expected with the pretreatment of the organoids with cytosine arabinoside.

The course of skeletal muscle differentiation in tissue culture is well established and, in most respects, follows the *in vivo* sequence, including the fusion of myoblasts into contractile myofibers expressing skeletal muscle-specific genes. Mechanical tension during organogenesis is an important organizing force in this tissue, and the application of *in vivo*-like mechanical forces to differentiating muscle cells *in vitro* leads to the formation of an organ-like muscle structure capable of performing directed functional work (Vandenburg *et al.*, 1991). Their use as a delivery system of bioactive molecules through genetic engineering has additional potential advantages over other forms of muscle gene therapy. Recent evidence indicates that direct gene transfer by plasmid DNA injection *in vivo* may be even more difficult in primates than rodents because the thicker extracellular matrix around *in vivo* primate myofibers may inhibit DNA uptake by the muscle cells (Jiao *et al.*, 1992). This problem would not exist for the organoid delivery technique because insertion of the foreign DNA occurs before connective tissue formation. Another advantage of the organoid technique is that only postmitotic cells containing foreign DNA are implanted, reducing potential problems associated with proliferating cell transformation and tumor formation (Vile and Russell, 1995). Finally, techniques are well established for the successful removal and reimplantation of human skeletal muscle organs during reconstructive surgery and may be useful for long-term human muscle organoid implantation into an intramuscular site where it could possibly become innervated.

In summary, this preliminary report describes a new method for the delivery of physiological levels of a bioactive compound from organized postmitotic myofibers that combines gene therapy and tissue engineering techniques. Although still facing major hurdles before clinically effective therapies are possible (Crystal, 1995), skeletal muscle delivery systems for gene therapy look promising for future development.

## ACKNOWLEDGMENTS

This research was supported by NASA grants NAGW-4674 and NAG2-914. We thank Drs. T. King, M. Sheff, and A. Rosmarin for reading the manuscript and L. Gifford for manuscript preparation.

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Received for publication June 24, 1996; accepted after revision September 3, 1996.